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**14. ABSTRACT**
Antiangiogenic therapy of cancers targets tumor blood vessels to deprive malignant cells of oxygen and nutrients. Therapy of human cancers has produced poorer results than therapy of mouse tumors, a disparity that may be explained by more extensive coverage of human tumor vessels (e.g. in breast cancers) by pericytes, which may be rendering vessels more therapy-resistant. Mouse mammary tumor virus (MMTV)-induced mammary carcinomas reproduce the extensive pericyte coverage of tumor vessels seen in human breast cancers and are relatively refractory to antiangiogenic therapy compared to other mouse tumors. This project attempts to decrease pericyte coverage of vessels in these and other tumors by manipulating activity of endothelial cell (EC) Tie2 receptors and improve tumor response to antiangiogenic therapy. We created K1735 tumors and transgenic mice that inducibly express Tie2Ex, an inhibitor of EC Tie2 activation, under doxycycline regulation. Dox-induced Tie2Ex expression in K1735 tumors reduces tumor vessel phospho-AKT and pericyte coverage and causes tumor EC death, vessel regression and tumor stasis. Using Tie2Ex to treat tumors in combination with an antiangiogenic agent, sorafenib, that reduces tumor vessel phospho-ERK expression displayed synergistic effectiveness. We created double transgenic mice that express Tie2Ex in mammary tissue under Dox regulation and are awaiting development of mammary carcinomas in these mice to study the effect of Tie2 inhibition in mammary tumors. We obtained a no-cost extension to finish this project.

**15. SUBJECT TERMS**
Angiogenesis, antiangiogenesis, experimental therapeutics

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**Principal Investigator:** Lee William M. F.
*Last Name* *First Name* *MI*

**Proposal Title:** Targeting Tie2 to Increase Breast Cancer Responsiveness to Antiangiogenic Therapy

## Introduction

Antiangiogenic therapy of cancers involves inhibiting tumor blood vessel development to deprive tumors of vital oxygen and nutrients. The potential benefits of antiangiogenic strategies have been dramatically shown in mouse tumor models. Results in human clinical trials, however, have been less striking. Recent trials have shown survival benefits, but tumor regression, which is often reported in murine tumors, is rarely seen in treated human cancers. A potential explanation for this disparity in treatment outcomes is that the vasculature of human tumors may be more resistant to antiangiogenic therapies. This may be due, at least in part, to extensive pericyte coverage of vessels in many common human cancers, such as breast cancers, compared to a relative deficiency of pericytes surrounding vessels in commonly studied mouse tumors. Pericytes are periendothelial mesenchymal cells that surround capillaries. Their presence is associated with microvessel maturity and stability and may confer resistance to certain antiangiogenic agents. Mouse mammary tumor virus (MMTV)-induced mammary carcinomas arising in C3H/HeN mice may more faithfully model human breast cancer vasculature inasmuch as vessels in these tumors have extensive pericyte coverage like in human breast cancers. Interestingly, we found these MMTV-induced mammary carcinomas were resistant to rIL-12 antiangiogenic therapy, which was effective against every other mouse tumor model tested (Lee JC et al., Canc. Res. 62: 747-755. 2002).

The endothelial-specific receptor tyrosine kinase, Tie2, regulates microvessel pericyte coverage, as well as activating endothelial cell (EC) signal transduction pathways that promote their survival (e.g. the PI3 kinase-AKT signaling pathway). Studies proposed in this IDEA award examine whether inhibiting Tie2 activation diminishes pericyte coverage and apoptosis-resistance in tumor vessels in transplanted mouse tumors and MMTV mammary tumors and enhance their susceptibility to antiangiogenic therapy. Our strategy was to develop an inducible system for inhibiting Tie2 activation by expressing the extracellular domain of Tie2 (Tie2Ex) as a decoy receptor in K1735 melanoma cells. These are easily transfectable tumor cells and produce tumors with well-characterized vasculature. Once the inducible system for Tie2Ex expression was validated in this system, we would introduce it into MMTV-induced mammary carcinomas using transgenic manipulation of mice.

*This grant was in a no-cost extension year in 2006-2007 because (a) grant funds had not been fully expended by the end of the third year of funding in 2006, and (b) work was ongoing due to a 1 year project delay in 2004 caused by an outbreak of mouse hepatitis virus (MHV) in our vivarium.*

## Body of Report

### *Task 1. Determine whether blocking Tie2 reverses pericyte coverage in K1735 tumors.*

#### *a. Develop K1735 cell line that inducibly expresses Tie2Ex*

The tetracycline (doxycycline)-inducible ("Tet-On") system was chosen for regulating expression in tumors of Tie2Ex, the soluble, extracellular domain of the Tie2 receptor that acts as a decoy receptor for Tie2 ligands and inhibits Tie2 activation. We transfected K1735 tumor cells with a plasmid (pEF2-rtTA) to constitutively express the reverse tetracycline transactivator (rtTA), which activates transcription of genes under the control of a tetracycline-response element (TRE) only in the presence of doxycycline (Dox). We identified a single clone of transfected K1735 cells, K1735.m39, with robust rtTA expression and supertransfected it with a TRE-Tie2Ex plasmid to generate a clone of doubly transfected cells, K1735.Tie2Ex$^{ind}$, that secreted virtually no Tie2Ex in the absence of Dox in the culture medium and secreted abundant Tie2Ex when 1 μM Dox was present. K1735.Tie2Ex$^{ind}$ cells secreted intermediate levels of Tie2Ex when lower concentrations of Dox (e.g. 0.1 μM) was added and shut off Tie2Ex expression when Dox was removed. The Tie2Ex secreted by these cells was shown to bind the Tie2 ligand, Ang1, and to inhibit Ang1 activation of Tie2 in EAhy926 endothelial cells. Control K1735.m39hygro$^{r}$ cells did not express Tie2Ex with or without Dox.

***b. Determine effect of Tie2Ex on vessels in K1735 tumors.***

K1735.Tie2Ex$^{ind}$ cells injected into syngeneic C3H/HeN mice formed tumors. Dox (2.0 mg/ml) was placed in the drinking water of the mice when their tumors reached 6-8 mm diameter, and tumors were removed for analysis at various days post-Dox. Western blot analysis of tumor lysate showed that K1735.Tie2Ex$^{ind}$ tumors from mice given 1.0 mg/ml Dox in their drinking water had abundant Tie2Ex, while those from mice not given Dox had no detectable Tie2Ex. Control tumors had no Tie2Ex whether or not their host was given Dox. Dox virtually arrested growth of K1735.Tie2Ex$^{ind}$ tumors but did not affect growth of control tumors. Tumors after 1 or 2 days of Dox had increased endothelial cell apoptosis detected by TUNEL staining and small focal areas of early necrosis; after 7 days of Dox, tumors had evident areas of necrosis mixed with areas of viable tumor (Figure 1); after 14 days of Dox, tumors were mostly necrotic. Control tumors treated with Dox grew progressively and did not show these changes. Thus, Tie2Ex expression results in tumor endothelial cell death and vessel regression that produces tumor necrosis over time. In apparently healthy regions of K1735.Tie2Ex$^{ind}$ tumors treated with Dox for 7 days, there was no significant change in the mean vessel density ($p>0.05$), but pericyte coverage of vessels was decreased (30% of vessels in –Dox tumors vs. 14% of vessels in +Dox tumors, $p<0.01$). Thus, pericyte coverage of vessels was reduced by Tie2Ex in K1735 tumors. We also studied the effects of Tie2Ex on vascular endothelial cell proliferation (Ki67) and signaling through the Raf-MEK-ERK and PI3K-AKT pathways. Vessel Ki67 was unchanged, suggesting that angiogenesis was not inhibited. pERK was unchanged, suggesting that signaling through the Raf-MEK-ERK pathway was not inhibited. pAKT was decreased after only 1-2 days of Dox, indicating that Tie2Ex inhibited signaling through the PI3K-AKT pathway even before it caused a decrease in vessel pericyte coverage. We are currently examining the relationship between vessel pericyte coverage, endothelial pAKT and apoptosis. The findings in K1735 tumor justified proceeding with our plans to test the effect of Tie2Ex expression in MMTV-induced mammary carcinomas by creating transgenic mice.

***Task 2.*** **Determine Tie2Ex effects on vessels in MMTV-induced breast tumors.**

***a. Develop transgenic mice that inducibly express Tie2Ex.***

The Transgenic Mouse Facility at Penn generated C3H/HeN TRE-Tie2Ex transgenic mice using the TRE-Tie2Ex plasmid used to transfect K1735 tumor cells. Mouse lines that stably transmitted the transgene were identified and crossed with MMTV-rtTA transgenic mice of the FVB strain (provided by Lewis Chodosh, University of Pennsylvania), which express rtTA in mammary and certain other tissues. Double transgenic (MMTV-rtTA$^{+}$, TRE-Tie2Ex$^{+}$) F1 female progeny showed high-level Dox-inducible Tie2Ex expression in mammary glands and certain other organs (e.g. salivary glands) (Figure 2).

***b. Develop MMTV-infected female mice that will develop mammary tumors that express Tie2Ex under Dox induction.***

To generate mice with MMTV-induced tumors that carry the MMTV-rtTA and TRE-Tie2Ex transgenes, we crossed FVB MMTV-rtTA male transgenic mice with C3H/HeN TRE-Tie2Ex female transgenic mice that were infected with mouse mammary tumor virus. As both transgenic lines are heterozygous and only female mice develop MMTV-induced tumors, only 1 in 8 pups were double transgenic females. These were infected with MMTV virus transmitted via mother's milk transmission and beginning at 6 weeks of age were force bred until 5-6 months of age to enhance MMTV mammary tumorigenicity. The oldest of the retired breeder, double transgenic mice are now 9-10 months old, but none have yet developed tumors. Once tumors develop in these mice, fragments of these tumors will be transplanted into SCID recipient mice to passage and expand these tumors for study. A potential explanation for the delay in mammary tumor formation is that these mice are C3H/HeN x FVB F1 and may develop tumors with different kinetics than C3H/HeN mice.

***c. Determine if transgenically expressed Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors.***

***Task 3.*** **Determine if Tie2Ex increases responsiveness of MMTV-induced breast tumors to antiangiogenic therapy.**

This study has not been initiated because MMTV-induced mammary carcinomas that inducibly express Tie2Ex have not yet arisen (Task 2b and c). However, we did study the effect of Tie2Ex expression on response to antiangiogenic agents using K1735.Tie2Ex$^{ind}$ tumors. Induction of Tie2Ex expression in tumors

when they are 2-3mm in diameter results in marked slowing of tumor growth. However, induction of Tie2Ex when tumors are 5-6mm in diameter fails to slow growth. Because Tie2Ex induction decreases endothelial PI3K-AKT signaling (represented by pAKT) but no change in Raf-MEK-ERK signaling (represented by pERK), we tested the effect of combining sorafenib and Tie2Ex therapy. Sorafenib is a small molecule multikinase inhibitor that was designed to inhibit Raf kinase but has been shown to inhibit VEGFR2 in vitro. However, several lines of evidence obtained in our lab suggest that sorafenib inhibits tumor angiogenesis not by inhibiting VEGFR2 but by inhibiting Raf kinase in endothelial cells. Like Tie2Ex, sorafenib treatment inhibited growth of 2-3mm K1735 tumors but did not inhibit growth of larger 5-6mm tumors. These larger tumors, which are not controlled by either Tie2Ex or sorafenib, were well controlled by the combination of the two therapies (Figure 3). These results show that therapeutics may be combined using information about their pharmacodynamics to achieve better therapeutic effect. We are currently analyzing why combined therapy is more effective than single agent therapy.

**Key Research Accomplishments**

We created K1735.Tie2Ex$^{ind}$ cells and tumors that expression Tie2Ex under Dox-induction.

Discovered that inhibition of Tie2 signaling in tumor vessels results in decreased endothelial signaling through the PI3 kinase-AKT pathway (represented by phospho-AKT) but does not affect signaling through the Raf-MEK-ERK pathway (represented by phospho-ERK).

Inhibition of Tie2 signaling in tumor vessel induces their regression (represented by vessel TUNEL staining) and tumor necrosis, indicating that this is a potentially useful antiangiogenic therapeutic approach. Tie2 inhibition, however, does not appear to affect formation of new vessels or tumor angiogenesis (represented by vessel Ki67 staining).

Tumor therapy combining Tie2 inhibition with therapy with sorafenib, a Raf kinase inhibitor, appears to act synergistically and result in growth control of tumors that are not controlled by treatment with either agent alone.

We have created double TRE-Tie2Ex x MMTV-rtTA transgenic mice infected with MMTV which show robust Dox-induced Tie2Ex expression in their mammary glands.

**Reportable Outcomes (Publication bibliography)**

A manuscript is being prepared based on work done on K1735.Tie2Ex$^{ind}$ cells and tumors and will soon be submitted.

Personnel receiving pay: None (in no-cost extension year)
In past years: Jeff Tsai (graduate student), Stacey Hultine (research specialist), William Lee (PI)

**Conclusions**

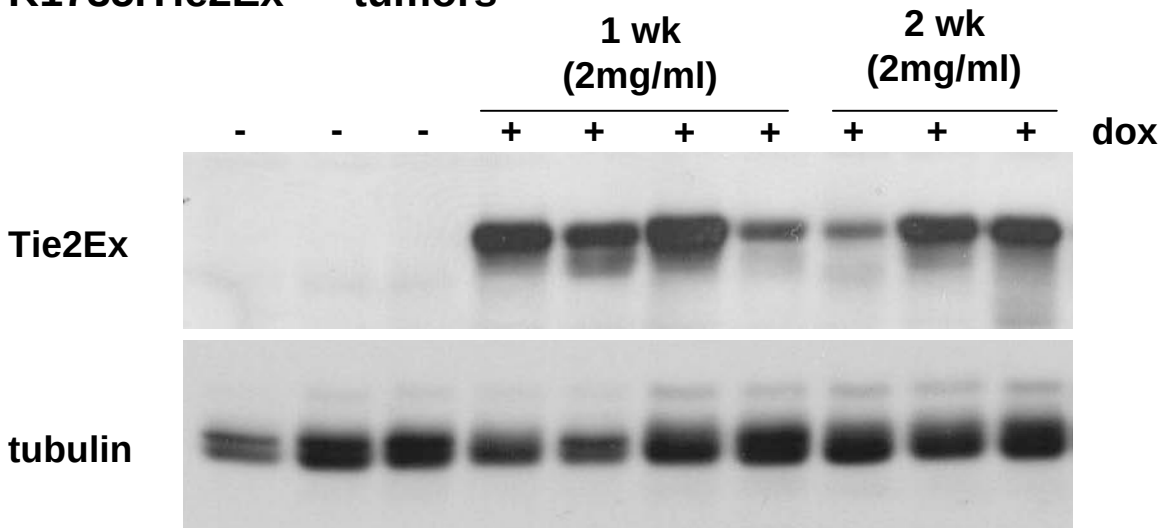
Expression of Tie2Ex in tumors resulting in inhibition of Tie2 activation inhibits endothelial signaling through the PI3K-AKT pathway and induces tumor endothelial cell death, tumor vessel regression and tumor growth arrest. Tie2Ex acts synergistically with sorafenib, a Raf-MEK-ERK pathway inhibitor, to control tumor growth.

**References and Appendices**

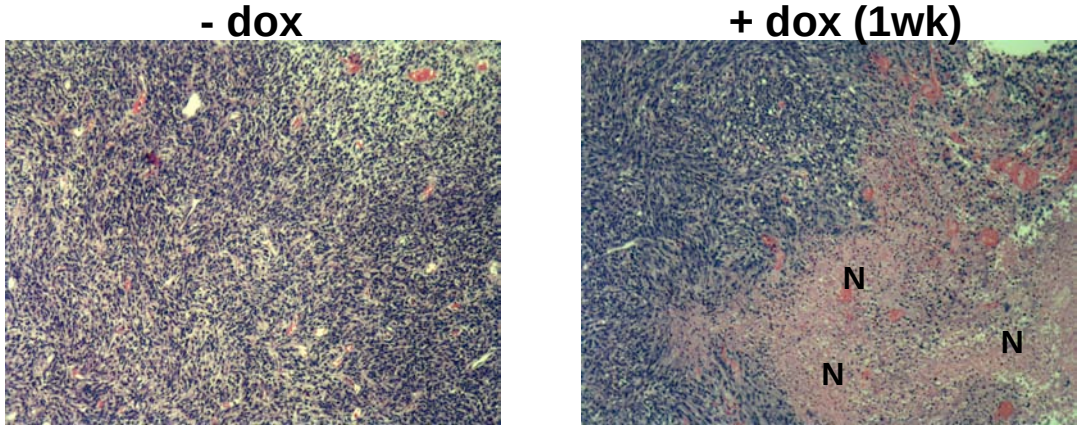
Presentation P19-9 at Era of Hope meeting, Philadelphia, PA (6/8/05 - 6/11/05).

**Figure 1. Inducible Tie2Ex expression and effects of expression in K1735.Tie2Ex$^{ind}$ tumors**

**A.**

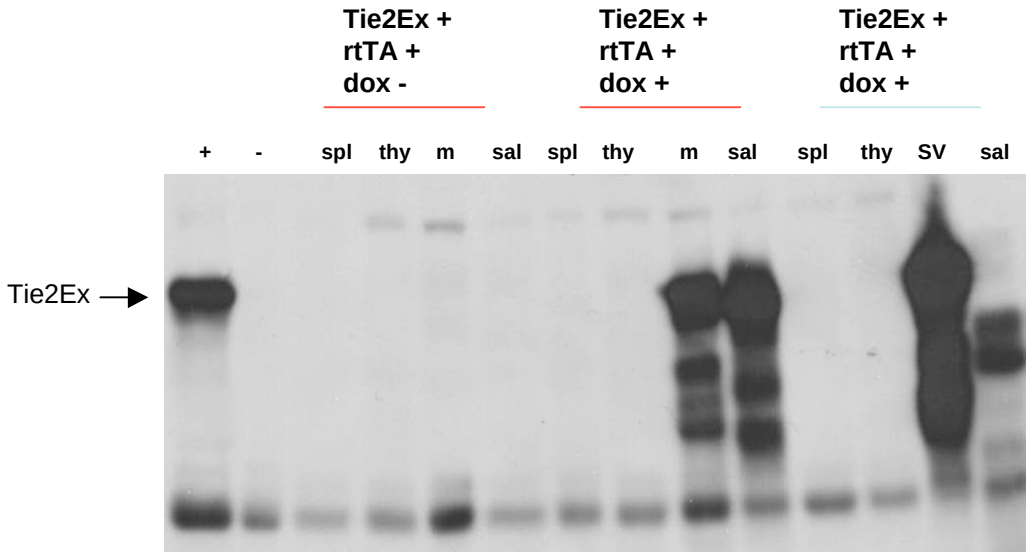



**B.**




***Legend*** (A) Anti-Tie2 antibody western blot of K1735.Tie2Ex$^{ind}$ tumor lysate taken from untreated mice or mice given Dox in their drinking water for 1 or 2 weeks; the blot was stripped and probed with anti-tubulin antibody to determine Lane loading. (B) H&E-stained tumor sections from an untreated mouse or a mouse given Dox for 1 week (N = necrosis).

## Figure 2. Inducible expression of Tie2Ex in mammary glands of MMTV-rtTA x TRE-Tie2Ex double transgenic mice




***Legend*** Organs were removed from MMTV-rtTA x TRE-Tie2Ex double transgenic mice treated (dox+) or not treated(dox-) with doxycycline (2mg/ml) in their drinking water for 2 days. Spleen (spl), thymus (thy), mammary glands (m), salivary glands (sal) and seminal vesicles (SV) were examined for Tie2Ex expression by SDS-PAGE/western blotting with anti-Tie2 antibody. Positive (+) and negative (-) Tie2Ex control samples were lysates fromTie2Ex-transfected and parental K1735 cells, respectively. Note dox-induced expression of Tie2Ex in mammary and salivary glands and in seminal vesicles in males.

# Figure 3. Growth of tumors treated with Tie2Ex, sorafenib or Tie2Ex+ sorafenib